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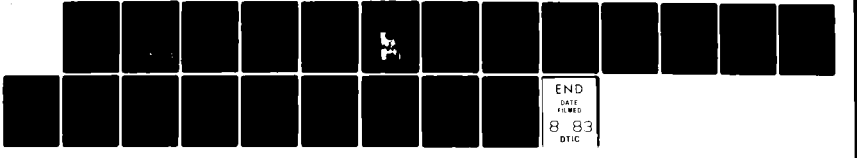

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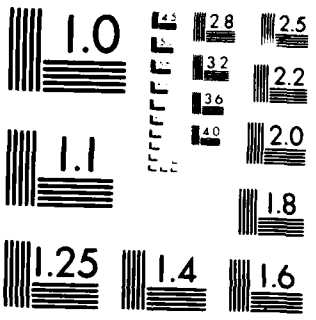

NRL Report 8688

Exposure of Creosote-Naive and Creosote-Conditioned *Limnoria tripunctata* (Menzies) to Untreated and Creosote-Treated Wood

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June 13, 1983

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Creosoted timbers are often destroyed in a few years by the marine crustacean wood borer, *Limnoria tripunctata.* Previous investigators have speculated that the ability of this animal to survive in creosote is afforded by hydrocarbon-metabolizing bacteria present on the wood surface, in the borer tunnels, or in the digestive tracts of the animals. This report evaluates the behavioral responses of two separate populations of *L. tripunctata* to creosoted wood. The health of the organisms was judged by measuring the production of fecal pellets and by percent survival.

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20 ABSTRACT (Continued)

A creosote-naive (never exposed to creosote) population of *L. tripunctata* exhibited 95% survival and produced numerous fecal pellets when exposed to untreated wood. Its survival on wood from an aged creosoted dolphin (dolphin wood) was slightly less, but numerous fecal pellets were produced by Day 2, indicating that this population began boring immediately. This rapid adaptation to the dolphin wood could be attributed to a decrease in the effectiveness of the residual creosote left in the surface layer of the wood. When this wood was sterilized, survival dropped to 80% and fecal pellet production fell below normal; when it was first scraped to expose a new surface, survival declined to 17% on unsterilized wood and 3% on sterilized wood. Fecal pellets were also scarce, indicating the undesirability of this wood to these laboratory-reared animals.

A creosote-conditioned population of limnorians collected from the same dolphin fared better than the creosote-naive individuals when exposed to creosoted wood. Survival and fecal pellet production were excellent when this population was exposed to: (a) untreated pine, (b) sterilized or unsterilized dolphin wood, or (c) unsterilized, scraped dolphin wood. When the scraped wood was first sterilized, however, survival dropped to 60% and fecal pellet production was much reduced, indicating an unfavorable response by these limnorians to an environment void of living organisms.

Chromatograms of fecal pellet extracts indicated that both populations of limnorians used or modified some of the lower molecular weight hydrocarbons present on the surface of the creosoted piling.

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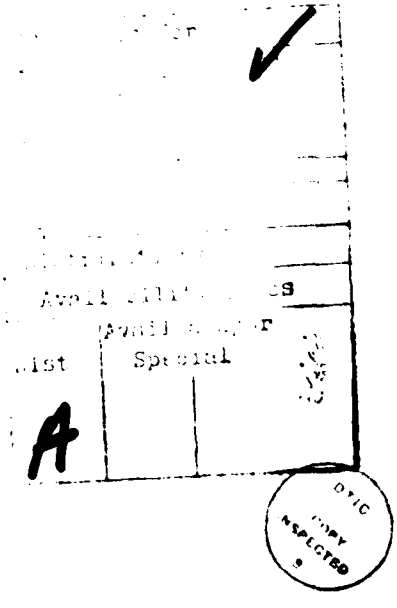

EXPOSURE OF CREOSOTE-NAIVE AND CREOSOTE-CONDITIONED *LIMNORIA TRIPUNCTATA* (MENZIES) TO UNTREATED AND CREOSOTE-TREATED WOOD

INTRODUCTION

The U.S. Navy has large quantities of wood in marine service such as piers, wharves, dolphins (bound clusters of free-standing piles), and fender systems. This wood must be treated to protect it from wood-destroying organisms. However, current wood preservatives are not entirely satisfactory. The most widely used treatments are impregnation with creosote or creosote/coal tar, but in tropical or subtropical waters these treatments do not protect wood from the crustacean isopod *Limnoria tripunctata* [1-4]. The waterborne preservatives (chromated-copper-arsenate and ammoniacal-copper-arsenate) are effective, but wood treated with these preservatives tends to lose its mechanical strength [5,6], and treated piling may fracture when impacted by a ship's hull (Fig. 1).

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Fig. 1 — Piling treated with copper-chrome-arsenate, fractured after impact by a ship's hull

Manuscript approved December 22, 1982

The Naval Research Laboratory (NRL) is interested in determining the biological defense mechanism(s) used by *L. tripunctata* to protect itself from the toxic effects of creosote. Identification of such a mechanism might suggest a means of improving the performance of this widely used preservative so that it would also be effective against crustacean as well as molluscan wood-destroyers. The obvious immunity of this species to creosote could be provided by diverse pathways. Among these are: (a) the preservative could be detoxified by creosote-metabolizing bacteria present on the wood surface and in the borer tunnels or existing commensally in the digestive tracts of the animals, (b) the glucose-permeable peritrophic membrane which encases material ingested by limnorians and isolates it from their intestinal linings could be impermeable to toxic creosote constituents keeping them contained within the peritrophic sac, and (c) creosote could activate an inducing agent which triggers the production by limnorians of special enzymes needed to metabolize the creosote. The first of these hypotheses is the most attractive and involves a relationship among microorganisms, creosote, and limnorians. Reported here are initial experiments regarding this trio which evaluate the behavioral responses of two limnorian populations with differing histories to creosoted wood.

Several investigators have studied the relationship between *L. tripunctata* and creosote. Becker and Schulze [7] impregnated pine blocks with creosote fractions of various boiling ranges and tested their efficacy toward limnorians. Sweeney, Price, and Miller [8] and Becker and Kampf [9] performed similar experiments but accelerated the leaching to determine long-term effects. Hochman et al. [3,10], Vind and Hochman [11], Roe [12], and Richards and Webb [13] evaluated the toxicity toward *L. tripunctata* of creosote, creosote/coal tar, inorganic compounds, organometallic compounds, and combinations of these chemicals with creosote. The results of these toxicity studies have consistently indicated a creosote tolerance by *L. tripunctata*.

Laboratory experiments have shown that the surfaces of creosoted piling contain microorganisms capable not only of metabolizing creosote, but also of producing chemical changes similar to those occurring to creosote exposed in the natural environment [14,15]. Microbial degradation of selected polynuclear aromatic petroleum components has also been studied [16]; and recently, researchers at the University of Maryland [17,18] determined the types of bacteria involved in the microbial succession occurring on naphthalene-enriched, creosoted wood.

Studies performed with laboratory-reared animals on untreated wood indicated a lack of resident microflora in the intestine of *L. tripunctata* [19-23]. By contrast, it was shown recently [24] that *L. tripunctata* associated with treated piling at Roosevelt Roads, Puerto Rico, did have microorganisms present in their digestive system, both within the peritrophic membrane which encases the ingested contents, and within the space between the peritrophic membrane and the intestinal lining. Zachery and Colwell [24] and Emery [25] suggested that possibly a commensal relationship exists between the gut-associated bacteria and the isopod. Many of these bacteria contained electron-transparent, cycloplasmic inclusions [24], typical of those seen in bacteria growing on naphthalene, hexadecane, or tetradecane as the sole carbon and energy source (26). Possibly, uptake and/or use of hydrocarbons by the gut-associated bacteria in *L. tripunctata* living in creosoted wood detoxified the creosote for the isopod. This could explain the severe damage to creosote-preserved wood structures in tropical waters.

MATERIALS AND METHODS

Animals

Standardized laboratory cultures of *L. tripunctata* are needed to establish the degree of experimental control necessary for quantitative, reproducible work. Two such cultures are maintained at NRL. One population of laboratory-reared animals, which will be referred to as *creosote-naive*, was acquired from Dr. Ruth Turner (Harvard University) and has since been maintained through multiple generations on untreated southern pine in a closed, all-glass, recirculating saltwater system described by Parrish and Bultman [27].

A second, more recent, population was obtained from the remains of an approximately 35-year-old, creosoted pine dolphin located near the pier of the NRL Marine Corrosion Laboratory (MCL), Key West, Florida. This native stock referred to as *creosote-conditioned* has been subsequently cultured in an aquarium system identical to that described above, on either wood acquired from the same pine dolphin (dolphin wood) or from unsubmerged sections of a creosoted fender pile of undertermined age from the Norfolk Naval Shipyard. Before being used in experiments, adult specimens from either population were teased from their tunnels and kept for 48 hours in dishes of seawater, each containing a piece of Whatman #42 filter paper as a food source. Those animals that did not appear to be healthy at the end of this time were discarded.

Experimental Dishes and Seawater

All seawater was made up according to Kester et al. [28] in 95-liter (25-gallon) batches. All experiments were performed in 100 × 50 mm crystallizing dishes filled with 200 ml of this water. Daily transfer of limnorians and wood to fresh seawater minimized detrital buildup as well as the accumulation of creosote components leaching from the treated wood.

Wood

Control wood consisted of pine disks (5.2 cm diameter × 0.6 cm thick) or matchstick-size pieces (5.0 cm long × 0.5 cm wide × 0.2 cm thick) cut from untreated southern pine. Treated wood initially consisted of creosote-impregnated pine disks (also 5.2 cm diameter × 0.6 cm thick); later matchstick-size pieces from the dolphin wood (identical in size to the untreated pine specimens described above) were substituted for the larger creosoted disks. Pine disks were treated by impregnating them with Grade 1 (80-30-36) creosote obtained from Koppers Company. Impregnations were performed by using a modified Bethell full cell vacuum/pressure technique [29]; pickup of the preservative averaged 0.48 g/cm^3 (30 lb/ft^3). After impregnation the disks were air-dried for 4 days. Some of the treated disks were leached in the laboratory for 6 weeks in a specially designed trough using water from one of the culture tanks flowing at the rate of 90 ml/h (1 drop/20 s). The remainder of the treated disks was leached in Florida Bay (ocean water) for 11 weeks at MCL.

Initial experiments showed that neither population of limnorians could tolerate exposure to the treated disks, even those previously leached for 11 weeks in seawater, apparently because of a rapid accumulation of toxic creosote components in the restricted volume of water in the crystallizing dishes. Subsequently, matchstick-size pieces of dolphin wood were used.

Gas Chromatograph

Creosote used in these experiments was characterized by gas chromatography. Treated wood specimens were extracted with chloroform, and the extracts were analyzed on a Varian Aerograph, Model 2700, dual column chromatograph equipped with a flame ionization detector. This chromatograph employed a matched pair of 3 mm (1/8 in.) diameter, 152 cm (5 ft) stainless steel columns packed with Se 30 on 100/120 mesh Chromosorb G-PH. The signal output from the chromatograph was processed by a Texas Instruments Servo/riter II strip chart recorder which recorded the spectrographic signatures of the extracts. For all samples the columns were temperature-programmed for 50°C to 300°C with a linear temperature change rate of 6°/min and with the detectors and injectors at 200°C. The carrier gas was helium flowing at the rate of 25 l/min; hydrogen and airflow rates were 30 and 300 ml/min, respectively. Injections were made with a 5 μl syringe. Calibrations were made with a qualitative standard consisting of a reference mixture of pure compounds.

EXPERIMENTAL DESIGN

Exposure of Creosote-Naive Limnorians to Creosoted Wood

Creosote-naive limnorians were exposed to creosoted pine disks leached at NRL and to untreated pine control disks. Ten adult limnorians were placed in each of four crystallizing dishes. Each of two control dishes contained a disk of untreated pine; each of two experimental dishes contained a pine disk impregnated with creosote. Behavior and survival data were collected for 10 days.

Exposure of Both Populations to Creosoted Wood

Both populations of limnorians were exposed to treated pine disks leached either at NRL or MCL, untreated pine disks, and treated matchstick-size pieces from the dolphin wood. The experimental setup is outlined in Table 1.

Table 1 — Exposure of Both Populations to Creosoted Wood

Dish Designation	Population	Wood Description
A — CN1	Creosote-naive (CN)	A = Dolphin wood (matchstick-size pieces)
2		
CC1	Creosote-conditioned (CC)	
2		
B — CN1	Creosote-naive (CN)	B = Key West leached creosote pine (circular disks)
2		
CC1	Creosote-conditioned (CC)	
2		
C — CN1	Creosote-naive (CN)	C = Lab leached creosoted pine (circular disks)
2		
CC1	Creosote-conditioned (CC)	
2		
D — CN1	Creosote-naive (CN)	D = Untreated pine controls (circular disks)
2		
CC1	Creosote-conditioned (CC)	
2		

To determine if compositional differences existed between the residual creosote in the dolphin wood and the fresh creosote (Koppers) in the treated disks, chloroform extractions were performed on the wood from each source, and their chromatograms were compared before the experiments began. Survival and behavior data were collected for 26 days. Survivors were removed from their tunnels and counted, and their digestive systems were examined for bacteria by Dr. Arthur Zachery (University of Maryland Medical School).

Exposure of Both Populations to Untreated Wood, Unscraped Creosoted Wood, and Scraped Creosoted Wood

The purpose of this exposure was to compare the performance of both populations of limnorians when presented with sterilized and unsterilized wood, creosote-treated and untreated. In addition, surfaces of some of the treated wood were scraped before autoclaving to remove creosote possibly altered chemically by surface bacteria and to generate fresh surfaces. Table 2 outlines the experimental setup. Limnorian activity was measured by the onset of fecal pellet production and by overall survival (percent) at the end of 8 days.

Table 2 — Exposure of Both Populations to Sterile and Unsterile, Treated and Untreated Wood

Dish Designation	Population	Wood	
		Type	Sterility
CNSP 1,2	Creosote-naive (CN)	Untreated Pine (P)	Sterile (S)
CNUP 1,2			Unsterile (U)
CNSC/Un 1,2		Creosoted (C) Pine Unscraped (Un)	Sterile (S)
CNUC/Un 1,2			Unsterile (U)
CNSC/Sc 1,2		Creosoted (C) Pine Scraped (Sc)	Sterile (S)
CNUC/Sc 1,2			Unsterile (U)
CCSP 1,2	Creosote-conditioned (CC)	Untreated Pine (P)	Sterile (S)
CCUP 1,2			Unsterile (U)
CCSC/Un 1,2		Creosoted (C) Pine Unscraped (Un)	Sterile (S)
CCUC/Un 1,2			Unsterile (U)
CCSC/Sc 1,2		Creosoted (C) Pine Scraped (Sc)	Sterile (S)
CCUC/Sc 1,2			Unsterile (U)

After each daily transfer, the fecal pellets produced by the limnorians in each dish were collected by a drawn-out Pasteur pipet connected to a piece of surgical tubing with an attached plastic mouthpiece. Pellets collected from each dish were stored in separate vials after rinsing three times with sterile seawater to remove undigested wood components and to discourage bacterial decomposition of the pellets while in storage. At the end of the 4-day collection period and just prior to extraction with chloroform, the stored pellets were rinsed three times with distilled water to remove residual seawater. Chromatograms of the extracts were prepared.

Wood was sterilized by autoclaving at 6.8 kg (15 lb) pressure (121°C) for 15 min. After autoclaving, representative samples of wood were checked for sterility in a seawater-based basal medium (adjusted to pH 7) prepared according to Colwell et al. [30], containing glucose, casamino acids (an acid-hydrolized casein), and yeast extract. Since the limnorians were to be presented with this sterilized, creosoted wood, it was necessary to determine if autoclaving altered any of the chemical constituents of the creosote. Therefore, representative wood samples were extracted before and after autoclaving and the chromatograms of the extracts were prepared.

RESULTS AND DISCUSSION

Exposure of Creosote-Naive Limnorians to Creosoted Wood

Creosote-naive limnorians (Fig. 2(a)(b)) serving as controls exhibited a 95% survival after 10 days on a diet of untreated pine. By comparison, similar creosote-naive limnorians were completely intolerant to freshly creosoted disks (Fig. 2(c)(d)). Creosote components leaching into the water reached a lethal concentration very rapidly; within 48 hours, 75% of the original 20 animals had succumbed. An attempt to revive the remaining animals in fresh seawater was unsuccessful. Although the concentration of creosote components present in the water during any 24-hour period was not measured, the prevailing concentration when fatalities began to occur must have exceeded the 8 ppm of creosote reported by Hochman and Vind [31] to be lethal to limnorians.

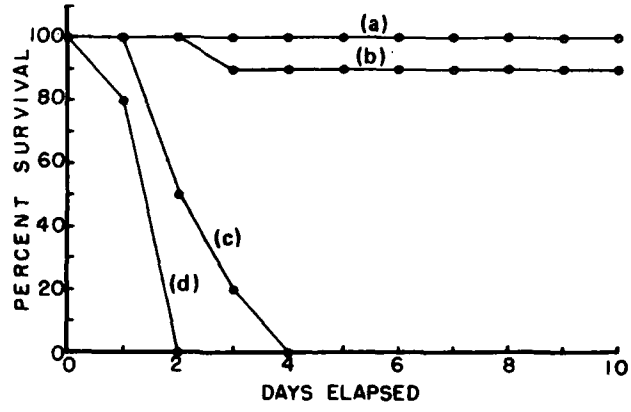

Fig. 2 — Exposure of creosote-naive limnorians (a)(b) to untreated pine and (c)(d) to freshly creosoted disks

These investigators also reported that the aromatic hydrocarbons present in creosote possess an affinity for nerve tissue which interferes with the normal metabolic processes of the animals and results in a depression of their central nervous systems. This phenomenon was evident within the first 24 hours of exposure and was characterized by a twitching of the animals' walking and swimming legs.

In the marine environment where potential hydrocarbon-utilizing bacteria abound and where creosote components leach from the wood into an infinite volume of constantly moving water, it takes about 4 days before these bacteria are found on freshly creosoted piling [19]. Thus, in certain respects, the subnormal survival of the laboratory reared animals was not surprising. Firstly, their restricted environment in the glass chambers remained static with water being changed only once every 24 hours. During this time the concentration of any creosote components leaching from the wood was constantly

increasing. Secondly, if creosote-naive limnorians harbor no bacteria in their digestive tracts [20-24], and if, in fact, hydrocarbon-utilizing bacteria do protect limnorians from the toxic constituents of creosote as Zachery and Colwell [24] suggest, then the laboratory-reared animals would perish before this protective flora could become established—assuming that the necessary bacterial forms were even present in the system. The basic point here, however, is that the early mortality of the limnorians in this exposure situation suggests that these animals do not have the capacity to generate their own compliment of induced hydrocarbon-degrading enzymes in response to the presence of creosote components, thus eliminating this mechanism (see page 1) as a means of protecting limnorians from creosote.

In subsequent experiments creosoted disks leached in natural seawater, and the smaller matchstick-size pieces of creosoted wood from the aged dolphin were used to minimize the buildup of toxic components of creosote in the experimental dishes.

Exposure of Both Populations to Creosoted Wood

A comparison of the chromatogram peaks of the extract from the dolphin wood (Fig. 3(a)) with those from fresh creosote (Fig. 3(b)) indicated that even when wood is exposed to seawater for many years, it still possesses the same major organic compounds as fresh creosote. This agrees with the results of experiments conducted by Lorenz and Gjovik [32] which showed that creosote, after a long exposure in seawater, can still have a composition similar to the original creosote, and that borer damage did not appear to be associated with the dissappearance of any particular component. Baechler and Roth [33] found 298 and 317 kilograms of oil per cubic meter of wood (18.6 and 19.8 pounds of oil per cubic foot of wood) still present in creosoted marine piling at Portsmouth, Virginia after 59 years of exposure, and Webb [34] cited other instances documenting the longevity of creosote in treated wood. Consequently, we concluded that variations in the relative concentration of the major components between the fresh creosote and that extracted from the dolphin wood were not significant enough to invalidate results comparing the life spans of limnorians exposed to the freshly creosoted disks with those limnorians exposed to the dolphin wood.

Table 3 compares the relative retention times of the major creosote peaks (see Fig. 3) measured on our columns with those of Nestler [35]. Before this comparison could be made, however, Nestler's relative retention values, which were based on a 200°C isothermal run, had to be converted to retention times and then relative retention times for our temperature-programmed columns. To do this one had to assume that: (a) on isothermal columns the $\log_{10}$ of the retention time was directly proportional to the number of carbon atoms; and (b) on temperature-programmed columns the retention time, itself, was also directly proportional to the number of carbon atoms, therefore the $\log_{10}$ of the isothermal retention time was directly proportional to the temperature-programmed retention time [36].

Based upon the retention time on our columns of naphthalene (8.2 min) and n-C_{20} (24.9 min), Table 3 shows that the measured relative retention values of two other standards, phenanthrene (0.80) and pyrene (1.04) differed by only 2.43 and 1.92%, respectively, from Nestler's predicted values of 0.82 and 1.06. As a result of this agreement, the remaining peaks were tentatively identified by comparison of the relative retention values of our actual measurements with predicted values calculated from the data in Nestler's Table II. The 15 polycyclic, aromatic hydrocarbons listed in Table 3 are typical of the major constituents in creosote; several investigators [35,37] have consistently identified them, and these constituents comprise almost 61% of whole creosote [37].

Data in Fig. 4 trace the survival of creosote-naive (short-dashed lines) and creosote-conditioned limnorians feeding on untreated and treated pine disks and on the treated matchstick-size pieces from the dolphin wood. Eighty percent of the creosote-conditioned and 95 percent of the creosote-naive individuals survived on the untreated pine control disks (Fig. 4(a)), and no significant behavioral differences were observed between these populations. On the other hand, 100% mortality occurred in

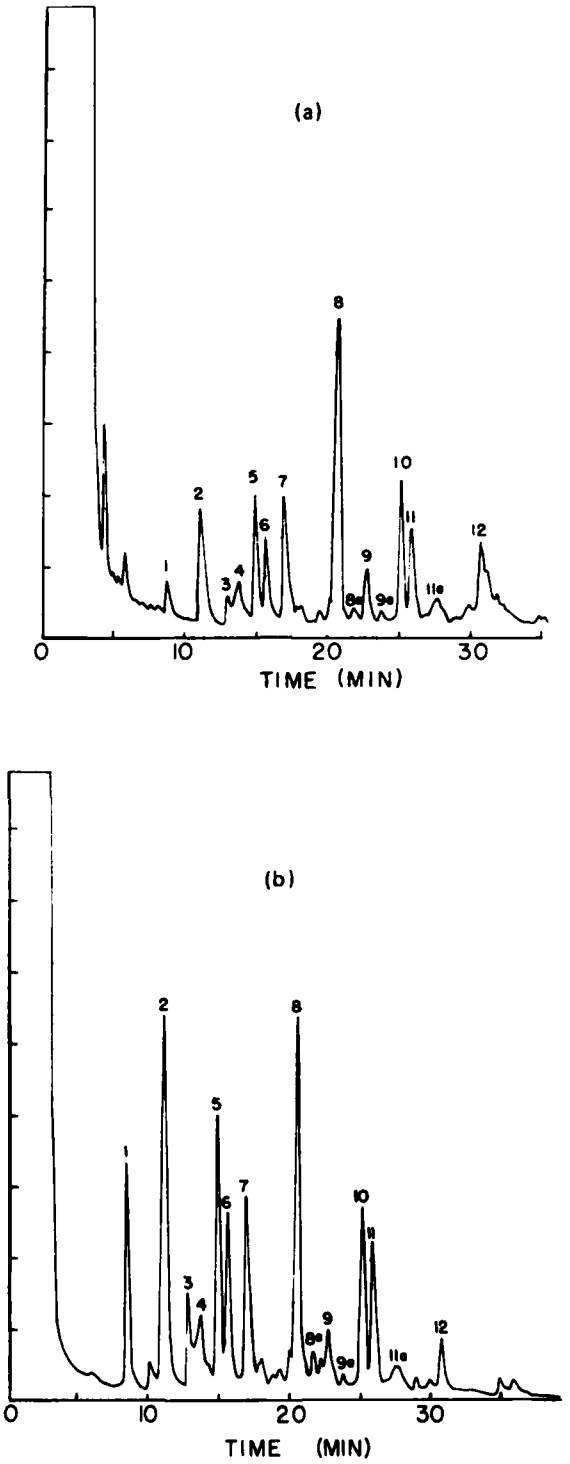

Fig. 3 — Chromatograms of (a) dolphin wood (about 35-years-old) and (b) fresh creosote. Numbers refer to individual peaks listed in Table 3.

Table 3 — Retention Times (RT) and Relative Retention Times (RRT) of Creosote Chromatographed on Se 30

Temperature Programmed[b]				Temperature Programmed[b]			
		Predicted Values		Experimental Measurements			
Peak No.	RRT[a] 200° Isothermal	Retention Time[c]	RRT Norm. nC_{20} = 1.00	Retention Time	RRT Norm. nC_{20} = 1.00	Figure 3 Tentative Identification[d]	Symbol
1	0.058	8.20	0.33	8.1	0.33	Naphthalene	N
2	0.086	10.45	0.42	10.4	0.42	2-Methylnaphthalene	2-MN
3	0.110	11.93	0.48	11.9	0.48	Biphenyl	Bi P
4	0.126	12.75	0.51	12.7	0.51	2,6-Dimethylnaphthalene	2,6-MN
5	0.170	14.50	0.58	14.5	0.58	Acenaphthalene	A
6	0.188	15.04	0.60	15.0	0.60	*Dibenzofuran*	*DBF*
7	0.238	16.53	0.66	16.4	0.66	Fluorene	F
8	0.463	20.44	0.82	20.4	0.82	Phenanthrene	P
8a	0.504	20.85	0.84	20.9	0.84	Carbazole	C
9	0.722	23.01	0.92	23.0	0.92	2-Methylantheracene	2-MAn
9a	0.834	23.82	0.96	23.8	0.96	9-Methylanthrocene/ 2-Phenylnaphthalene	9MA/2-PN
10	1.00	24.90	1.00	24.9	1.00	n-Eicosane	nC_{20}
11	1.31	26.52	1.07	26.5	1.06	Pyrene	Py
11a	1.89	28.68	1.15	28.7	1.15	2,3-Benzofluorene	2,3-BF
12	3.56	32.33	1.30	32.4	1.30	Chrysene	Ch

[a]Taken from Nestler Table II [27].

[b]50-300°C @ 6°/min.

[c]Conversions based on Nestler's RRT 200° Isothermal from Table II and the assumption that RT (Temp. Prog.) is directly proportional to $\log_{10}$ RRT (Iso). Conversion: $y = 13.51X + 24.9$, where $X = \log_{10}$ of RRT (Iso).

[d]Based on agreement of RRT of underlined standards with those of Nestler.

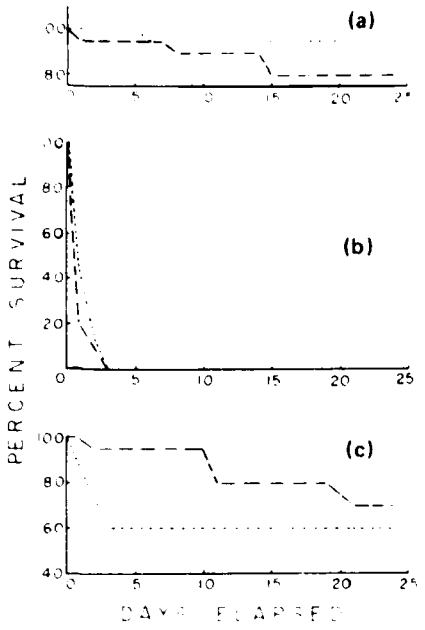

Fig 4 — Exposure of creosote-naive (short-dashed lines) and creosote-conditioned limnorians feeding on (a) untreated pine control disks, (b) freshly creosoted disks, and (c) matchstick-size pieces of dolphin wood

less than 72 hours when both the creosote-conditioned and creosote-naive animals were presented with freshly creosoted disks (Fig. 4(b)).

When both populations were exposed to the matchstick-size pieces of the dolphin wood (Fig. 4(c)), even though the difference in survival was minor, behavioral differences were observed. The creosote-conditioned animals began burrowing immediately, and black frass (a combination of excrement and undigested wood components) was produced in less than 24 hours. By comparison, the creosote-naive animals exhibited a 24-hour lag before burrowing activity was observed. By 36 hours, however, these animals were beginning to burrow, and by 48 hours the black frass, characteristic of animals feeding on creosoted wood, appeared. When the digestive tracts of creosote-naive limnorians were examined by electron transmission microscopy, bacteria commonly found in animals growing in creosoted wood were not found (although they did exist in the creosote-conditioned population).

Exposure of Both Populations to Untreated Wood, Unscraped Creosoted Wood, and Scraped Creosoted Wood

Gas Chromatograph Comparison of Autoclaved and Unautoclaved Wood

Chromatograms of extracts from four discrete samples, two each of unautoclaved and autoclaved creosoted wood, appear in Fig. 5. To determine if the autoclaving process altered the chemical constituents of the creosoted wood, the ratios of three representative peaks (1, 5, and 7) were compared. Peaks representing the lower molecular weight (lower boiling) hydrocarbons were chosen for comparison since these hydrocarbons would most likely be lost through the autoclaving process. Results presented in Table 4 indicate greater variation between the ratios of the peak heights of the unautoclaved control extracts (Fig. 5(a)(b)) than the autoclaved extracts (Fig. 5(c)(d)). These data imply that creosote in the autoclaved wood did not differ significantly from creosote in the unautoclaved wood. The broth cultures used to monitor the sterility of representative samples of the autoclaved wood remained uncontaminated throughout the experiment.

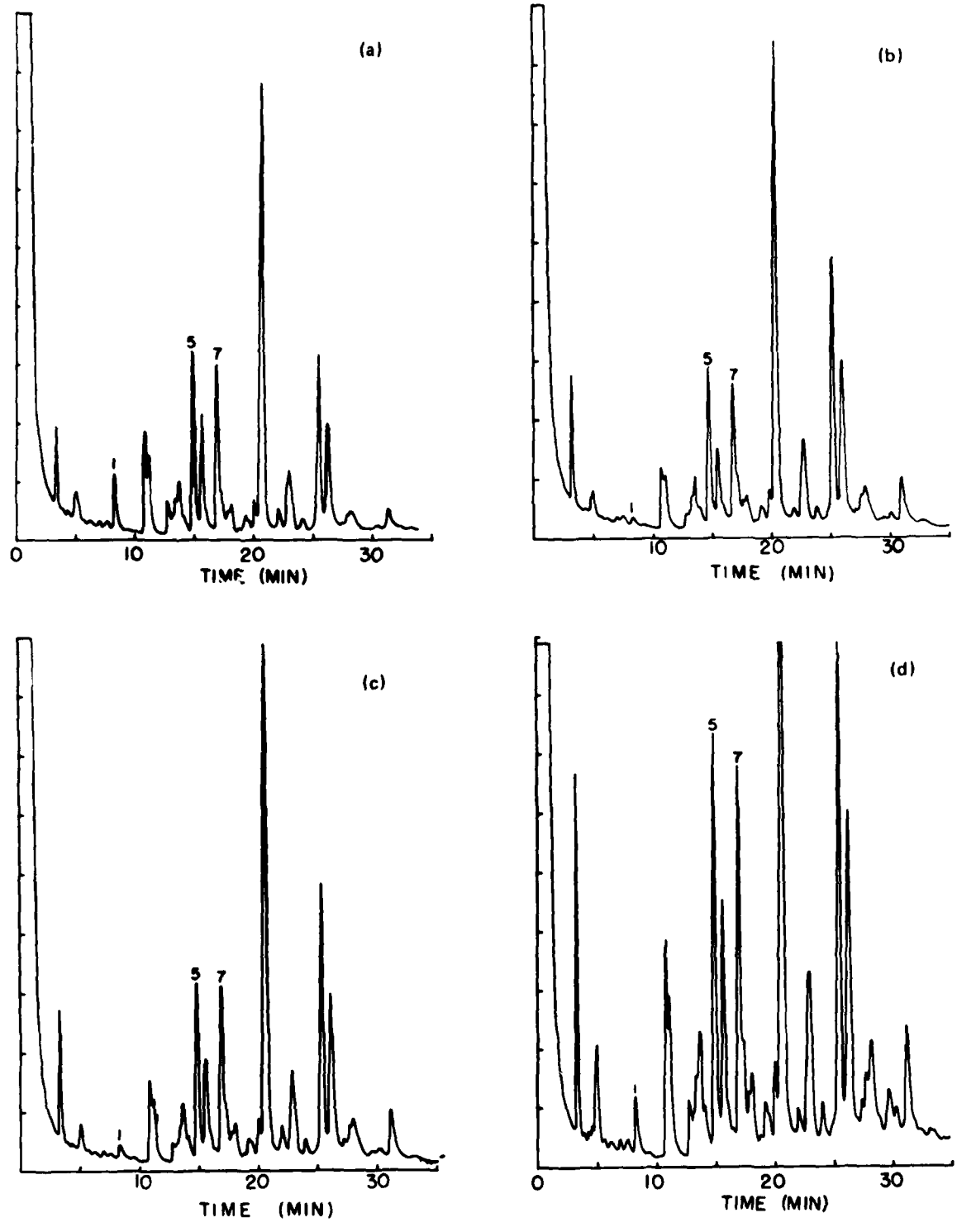

Fig. 5 — Chromatograms (a)(b) from two unautoclaved samples of creosoted wood and (c)(d) from two autoclaved samples of creosoted wood. Ratios of peaks 1, 5, and 7 are listed in Table 4.

Table 4 — Comparison of the Ratios of Peak Heights from Chromatograms (Fig. 5) of Unautoclaved and Autoclaved Aged Creosoted Dolphin (peak heights are presented in units)

Sample	Description	Height Peak 1	Ratio 1:5	Height Peak 5	Ratio 5:7	Height Peak 7	Ratio 1:7
A	Unautoclaved	9.25	0.32	29.0	1.07	27.0	0.34
B		1.00	0.04	25.5	1.11	23.0	0.04
C	Autoclaved	2.00	0.07	28.0	1.02	27.5	0.07
D		9.25	0.14	68.0	1.10	62.0	0.15

Exposure to Untreated Wood—Controls

Results of exposures of creosote-naive (short-dashed line) and creosote-conditioned limnorians to the unsterilized (Fig. 6(a)) and sterilized (Fig. 6(b)), untreated wood showed no difference in survival. Numerous fecal pellets were produced immediately by the creosote-conditioned as well as the creosote-naive animals.

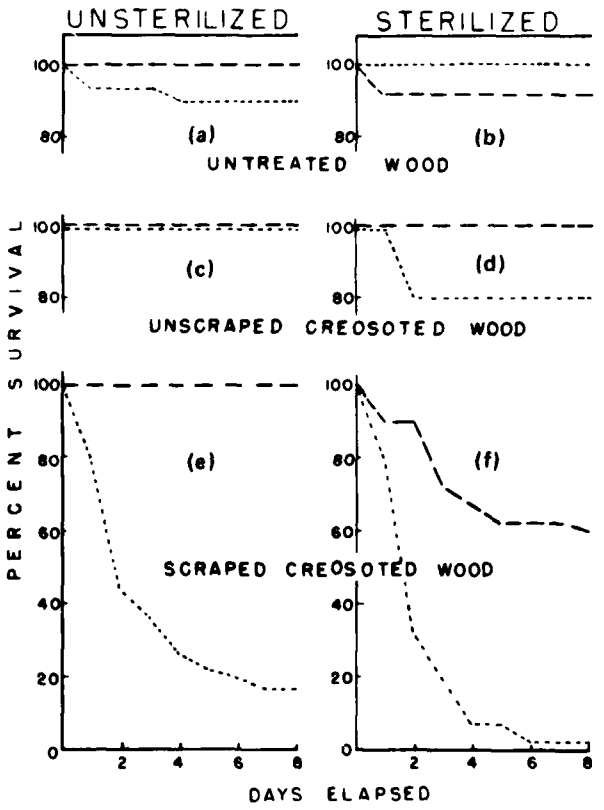

Fig. 6 — Exposure of creosoted-naive (short-dashed lines) and creosoted-conditioned limnorians (a)(b) to untreated wood; (c)(d) to unscraped, creosoted wood; and (e)(f) to scraped, creosoted wood

Exposure to Unscraped, Creosoted Wood

A comparison of the responses of both limnorian populations to sterilized and unsterilized, creosoted dolphin wood is presented in Fig. 6(c)(d). The 100% survival of the creosote-naive animals on the unsterilized wood was inconsistent with expectations. Not only did all animals survive, but their fecal pellet production was similar to that of the creosote-conditioned individuals. Assuming the premise of bacterial detoxification of creosote, the survival of the creosote-naive limnorians on the dolphin wood could be attributed to a decrease in effectiveness (before the animals contacted it) through bacterial degradation of the residual creosote left in the surface layer of the wood.

When sterilized wood was presented to both populations, a slight drop of 20% in the survival of the creosote-naive limnorians was seen; no change in survival was observed for the creosote-conditioned animals. In addition, only about 25% as many fecal pellets were produced by both populations as compared to fecal pellet production on unsterilized wood. Sterilization and the concomitant destruction of bacteria and/or fungi apparently produced a less appealing substrate to both groups of limnorians.

Exposure to Scraped, Creosoted Wood

Figure 6(e)(f) presents a comparison of the responses of both populations to scraped dolphin wood, sterilized and unsterilized. Creosote-conditioned animals (dashed line) exhibited 100% survival after 8 days of exposure and produced a normal amount of fecal pellets when provided with scraped, unsterilized, dolphin wood. Similarly treated wood was unacceptable to the creosote-naive limnorians (short-dashed line) as evidenced by their survival (17% after 8 days). In addition, no fecal pellets were produced by these limnorians for 48 hours. These differences in survival and fecal pellet production between the two populations are probably reflective of the fresh creosote surfaces produce through scraping. Survival and behavioral differences were more pronounced when the scraped wood was sterilized before being presented to both limnorian populations. Survival of the creosote-conditioned population dropped to 60% (as compared to 100% survival on unsterilized wood), and fecal pellets were not produced for the first 72 hours. This wood was completely unacceptable to the creosote-naive limnorians as evidenced by 3% survival (after 8 days). In addition, this population produced no fecal pellets until day 8.

These results indicate an unfavorable response by both populations of animals to an environment void of living microorganisms. The lag time observed before the limnorians commenced burrowing into the sterilized wood and producing fecal pellets probably indicates the time required for the bacterial population to become reestablished on the wood. These results support the contention of Ray [38] and Reynolds and Meyers [39] who suggest the importance of bacteria and fungi as a source of nitogren to limnorians in an environment where this protein-building nutrient is scarce. However, if the limnorians were dependent upon bacteria as a nutritional source only, the viability of these microorganisms should be unconsequential. Thus, these data also suggest that limnorians may be depending on the presence of bacteria for reasons other than nutrition, including bacterial detoxification of toxic creosote constituents.

A chromatogram of the extracts of fecal pellets from creosote-conditioned limnorians (Fig. 7(b)) indicates that these individuals used or modified some of the lower molecular weight hydrocarbons present on the surface of the dolphin wood (Fig. 7(a)). Chromatograms of extracts of fecal pellets from creosote-naive individuals also feeding on this wood were identical to that in Fig. 7(b). Peaks 1 through 8 (Fig. 3 and Table 3), representing compounds present in the creosote from the dolphin wood, were consistently missing from the fecal pellet chromatograms for both populations. Also shown is the chromatogram of untreated pine extract (Fig. 7(c)) as well as the resulting fecal pellet chromatogram from creosote-naive limnorians feeding on this pine (Fig. 7(d)). Chromatograms of extracts of fecal pellets from creosote-conditioned limnorians also feeding on untreated pine were identical to that

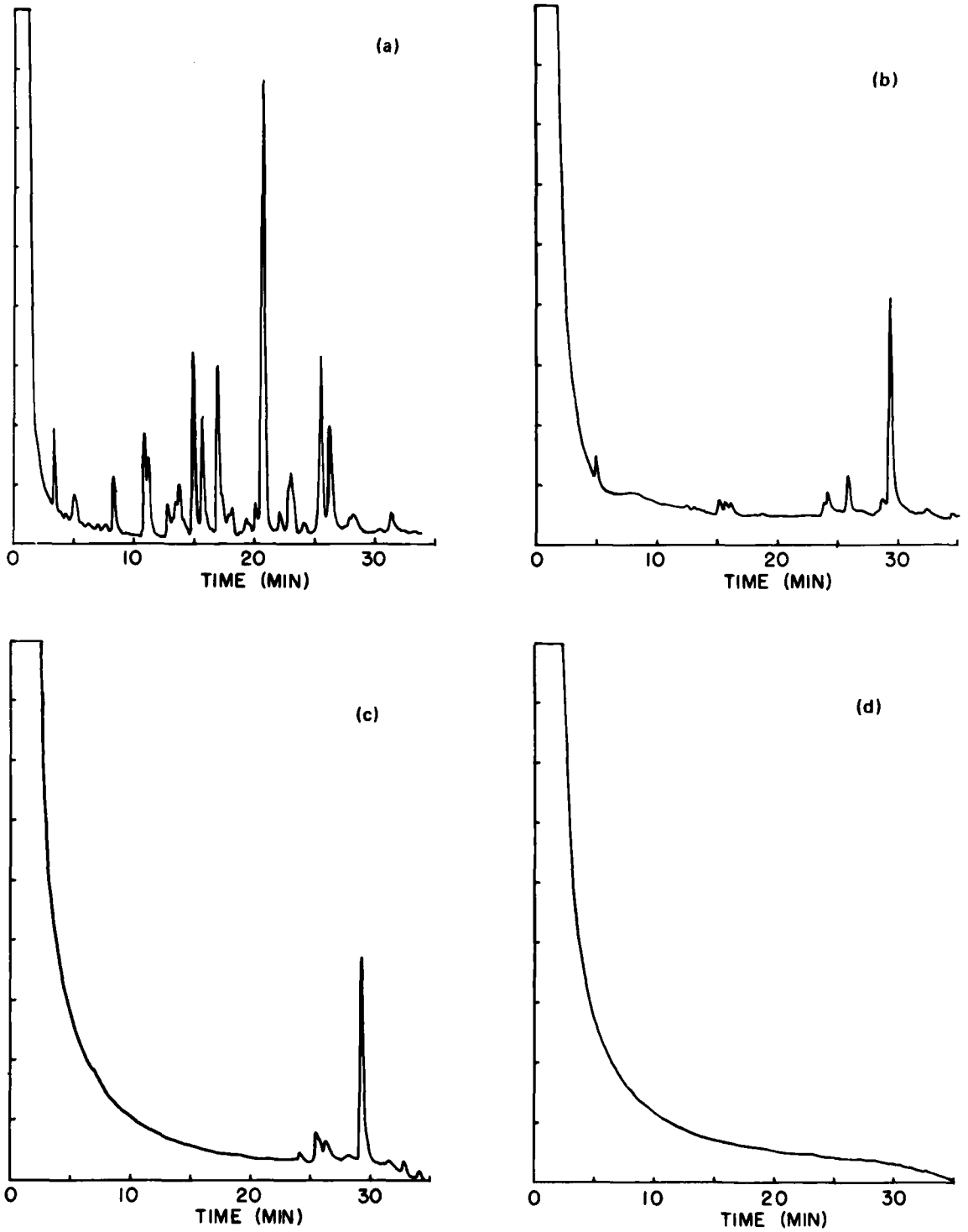

Fig. 7 — Chromatograms of extracts from the surface of (a) dolphin wood, (b) the fecal pellets from creosote-conditioned limnorians feeding on this substrate, (c) the surface of untreated wood, and (d) the fecal pellets of creosote-naive limnorians feeding on this substrate

in Fig. 7(d). The unidentified peaks present in extracts of the untreated pine were also missing on the fecal pellet chromatograms. These results raise some interesting speculations. If the toxic aromatic hydrocarbons in the creosote are being modified to less toxic forms, who or what is effecting this change? Zachery and Colwell [24] and Emery [25] have suggested gut-associated bacteria as the prime suspects, but exposure of *L. tripunctata* over the last 80 years to creosote in tropical environments could also have afforded them an immunity through genetic selection not possessed by their cousins from more northern climes.

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